

Effects of 4-Hydroxy-2-Nonenal on SERCA Pump Structure and
Function in Skeletal and Cardiac Muscle

by

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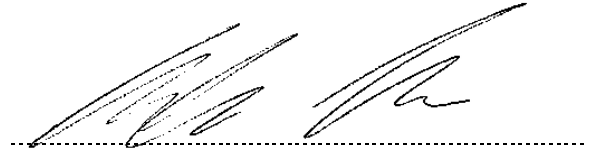
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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) pumps are approximately 100 kDa transmembranous proteins that catalyze the ATP-dependent transport of cytosolic Ca^{2+} [Ca^{2+}]_i into the sarcoplasmic reticulum (SR), thereby maintaining low concentrations of resting [Ca^{2+}]_i. Reactive free- and non-radical oxygen and nitrogen species regulate SERCA function. Additionally, free radicals can oxidize lipids producing bioactive lipid-peroxidation end-products which are capable of modifying membranous proteins, resulting in protein inactivation. Here, in order to characterize the effect of 4-HNE, a lipid-peroxidation end-product, on SERCA structure and function, mouse WG and LV tissues were treated with 4-HNE and subsequently assayed for maximal Ca^{2+} -dependent SERCA activity and SERCA post-translational structural modifications. Ca^{2+} -dependent, maximal SERCA activity assays demonstrate a dose-dependent functional impairment of the SERCA pumps following 4-HNE treatment; interestingly, western blotting revealed significant increases in 4-HNE – SERCA1a adduction. Moreover, blots illustrated that the SERCA inhibition by 4-HNE may be independent of nitrosative stress as there were no evident increases in total protein nitrotyrosine or SERCA specific nitrotyrosine formation with 4-HNE treatment. Additionally, there were no observed increases in total protein glutathionylation or SERCA specific glutathionylation with 4-HNE treatment. The impairments in SERCA function are likely due to increased SERCA aggregation as revealed by a significant reduction in monomeric SERCA in both isoforms, confirmed by results illustrating a significant increase in higher molecular weight SERCA aggregates. Immunoprecipitation of SERCA revealed reduced FITC binding to the SERCA1a isoform with no changes to the SERCA2a isoform, illustrating isoform specific structural changes following 4-HNE treatment. These results demonstrate that the reactive lipid-peroxidation end-product, 4-HNE, can directly impair SERCA function by potentially causing isoform specific structural changes.

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I have developed an arsenal of research experience both in and out of the lab, afforded the opportunity to travel and experience research in different contexts. Dr. A. R. Tupling, through his mentorship, has greatly progressed my skills as a researcher. I believe that his philosophy of being a supervisor, whereby he puts his students development first is extremely humbling, providing unconditional mentorship and guidance to further the interests of others before his own. These morals that he conducts himself by are a rare trait and something that I've come to greatly appreciate. In fact, I must thank him for not only the research experience that I've gained, but also for a lot of the soft-skills that I've come to acquire throughout this process.

During my time in the lab, I was fortunate enough to work alongside Ian, Val, and Dan... incredible. These three individuals are remarkable, their persistence and dedication to their craft is second-to-none. Their attention to detail from start to finish: ensuring the experiments are performed to standard, understanding exactly how each experimental method works and its limitations, displaying their results in a concise and visually appealing manner, and lastly, reporting on their findings through an expert command of the literature and language is impressive to say the least. Their perseverance and persistence towards "the end-goal" is absolutely something that I want to replicate in my own interests.

Lastly, I of course want to thank Dr. E. Bombardier, Dr. J. Ouyang, previous lab members, and current lab members. Dr. E. Bombardier, Dr. J. Ouyang, and the current lab members are a big part of what made my time here at Waterloo enjoyable. The running jokes, that for some reason I always seemed to be the center of, were humorous for the most part... but always in good fun. The laughs, discussions, and experiences that I've had with everyone have been a joy; I only hope to find a similar atmosphere between colleagues in the future.

Dedication

I would like to dedicate this thesis to my family, friends, colleagues, and supervisor whose continuous mentorship and support has guided me throughout my life and academic career.

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List of Abbreviations

[Ca ²⁺] _i	- Intracellular calcium
4-HNE	- 4-hydroxy-2-nonenal
AA	- Arachidonic acid
ACR	- Acrolein
ATP	- Adenosine triphosphate
BCA	- Bicinchoninic acid
Ca	- Calcium
Ca ²⁺	- Ionic calcium
CICR	- Calcium-induced calcium-release
CPA	- Cyclopiazonic acid
CTL	- Control
Cys	- Cysteine
DHPR	- Dihydropyridine receptors
DMSO	- Dimethyl sulfoxide
EC coupling	- Excitation contraction coupling
FITC	- Fluorescein isothiocyanate
GLA	- γ -linolenic acid
GSH	- Glutathione
Hsp 70	- Heat shock protein 70
IP	- Immunoprecipitation
IRS-1	- Insulin receptor substrate 1
kDa	- Kilodalton
LA	- Linoleic acid
LV	- Left ventricle
MDA	- Malondialdehyde
MS	- Mass spectrometry
Na ⁺ /K ⁺ pump	- Na ⁺ /K ⁺ ATPase
N-domain	- Nucleotide binding domain
PUFA	- Polyunsaturated fatty acid
PVDF	- Polyvinylidene difluoride
RyR	- Ryanodine receptors
S.E.	- Standard error of the mean
SERCA	- Sarco(endo)plasmic reticulum Ca ²⁺ -ATPase
-SH	- Sulfhydryl
Sln	- Sarcolipin
SR	- Sarcoplasmic reticulum
T-tubules	- Transverse-tubules
VDCR	- Voltage-dependent calcium release
WG	- White gastrocnemius
ω -6	- Omega-6

Introduction

Calcium (Ca) is one of 118 known chemical elements and is the 5th most abundant element in the human body by mass (Wittmann, Lohse, & Schmitt, 2015). Aside from Ca being a key constituent in the skeletal bones of all vertebrates, ionic calcium (Ca^{2+}) participates in numerous cellular processes. As a ubiquitous cellular signaling molecule, Ca^{2+} participates in: basic signal transduction (Fedrizzi, Lim, & Carafoli, 2008), growth and death signaling (Decuyper et al., 2011; Li, Zhao, Wei, Zhao, & Chen, 2011), metabolic signaling (Bal et al., 2012; Bombardier et al., 2013; Gamu, Bombardier, Smith, Fajardo, & Tupling, 2014), and muscular contraction in skeletal and cardiac muscle (Gordon, Regnier, & Homsher, 2001). The ubiquitous role of Ca^{2+} as a signaling molecule has an associated complexity, whereby the movement of Ca^{2+} functions as a physiological signal for a multitude of cellular processes. Therefore, due to its complexity as a messenger, the movement and compartmentalization of Ca^{2+} must be finely tuned with proteins, acting as ion-channels and pumps regulating the movement of Ca^{2+} , and innate membranous structures, serving as a way to compartmentalize and store Ca^{2+} when it is not involved in cellular signaling.

The role of Ca²⁺ and the sarcoplasmic reticulum in excitation-contraction coupling

Briefly, skeletal muscle contraction involves a sequence of events that results in contraction-relaxation cycles and is collectively labelled *excitation-contraction (EC) coupling*. Initially, EC coupling begins with a depolarizing stimulus at the neuromuscular junction. Upon depolarization, propagation of the stimulus ensues and occurs through rapid signal transduction across the sarcolemmal membrane, eventually reaching the transverse-tubules (t-tubules). At the t-tubules, the change in voltage (depolarization) detected by the voltage-sensitive dihydropyridine receptors (DHPRs) causes the rapid release of Ca²⁺ from the sarcoplasmic reticulum (SR) store via the ryanodine receptors (RyRs), a process labelled voltage-dependent calcium release (VDCR). The release of Ca²⁺ through the RyRs is due to the physical coupling between the DHPRs and RyRs, collectively labelled the *calcium-release units*. The resulting increase in intracellular Ca²⁺ ([Ca²⁺]_i) initiates skeletal muscle contraction (Leong & MacLennan, 1998).

Contraction in cardiac muscle slightly differs from skeletal muscle in a few respects. Importantly, EC coupling in cardiac tissue is chemical

in nature, whereby during a cardiac action potential extracellular Ca^{2+} enters the cell through DHPRs and initiates a process known as calcium-induced calcium-release (CICR); Ca^{2+} entry from the extracellular compartment through the DHPRs stimulates the release of Ca^{2+} from the SR store, initiating cardiac muscular contraction (Bers, 2002). The significance of the SR in both of these muscle types is that it is the primary site of Ca^{2+} storage within skeletal and cardiac tissue. This makes it a key structure as it pertains to EC coupling, allowing for Ca^{2+} release and subsequent muscular contraction.

The role of the sarco(endo)plasmic reticulum Ca^{2+} -ATPase pumps in excitation-contraction coupling

As the SR plays an integral role in muscular contraction, it is also intimately involved in muscular relaxation, acting as a fast Ca^{2+} sink for the rapid removal of $[\text{Ca}^{2+}]_i$ and initiating muscular relaxation. In skeletal and cardiac muscle, relaxation is predominately regulated through the action of the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) pumps (Bers, 2002; MacLennan, Rice, & Green, 1997) which are directly embedded within the SR membrane. The transmembrane SERCA pumps are classified as auto-phosphorylatable ATPases (P-type ATPases) that

electrophoretically migrate at approximately 100 kDa. Structurally, SERCA pumps consist of 10 transmembrane helices (M1-M10), three cytoplasmic domains (actuator, nucleotide-binding and phosphorylation domains), and small luminal loops (Brandl, Green, Korczak, & MacLennan, 1986; Menguy et al., 1998; Toyoshima, Nakasako, Nomura, & Ogawa, 2000). Collectively, these domains catalyze the ATP-dependent transport of $[Ca^{2+}]_i$ into the lumen of the SR (MacLennan, Abu-Abed, & Kang, 2002). SERCA pumps are primarily responsible for regulating resting $[Ca^{2+}]_i$ concentrations and initiating muscular relaxation (Bers, 2002; Rossi & Dirksen, 2006; Tupling, 2009). Specifically, in skeletal muscle, the SERCA pumps sequester large $[Ca^{2+}]_i$ loads from the cytoplasm into the SR lumen and maintain the approximately 10,000-fold concentration gradient across the SR membrane ($\sim 0.1 \mu\text{M}$ in the cytosol and $\sim 1,000 \mu\text{M}$ in the SR lumen) at rest (Rasmussen & Barrett, 1984), roughly a 4 order of magnitude difference. This steep concentration gradient maintained by the SERCA pumps is the predominant driving force by which Ca^{2+} exits the SR through the RyRs to elicit muscular contraction.

SERCA pumps are regulated through redox-signaling

In vertebrates, three distinct genes encode SERCA pumps: SERCA1, SERCA2 and SERCA3 are encoded by *ATP2A1*, *ATP2A2* and *ATP2A3*, respectively, and alternative splicing generates multiple SERCA isoforms (SERCA1a,b, SERCA2a-c and SERCA3a-f) (Hovnanian, 2007). In adult striated muscle, two SERCA isoforms predominate, namely SERCA1a and SERCA2a (East, 2000). SERCA1a is expressed almost exclusively in adult fast-twitch skeletal muscle fibres whereas SERCA2a is expressed both in cardiac and slow-twitch skeletal muscle (Wu & Lytton, 1993). SERCA1a is 994 amino-acids long while SERCA2a is 997 amino-acids and has approximately 84% identical amino-acid sequence to SERCA1a (Brandl et al., 1986). Several amino-acids critical to SERCA function have been identified through mutagenesis studies (Andersen, Clausen, Einholm, & Vilsen, 2003; Clausen & Andersen, 2003; Clausen, McIntosh, Vilsen, Woolley, & Andersen, 2003; MacLennan, Clarke, Loo, & Skerjanc, 1992; MacLennan et al., 1997; McIntosh et al., 2003; Menguy et al., 1998), some of which have been identified as primary targets for oxidative modification through carbonylation, nitrosylation, and glutathionylation in both mass-spectrometry (MS) (Schöneich & Sharov, 2006) and labeling (Fu & Tupling, 2009; Tupling et

al., 2004) experiments. Interestingly, studies have demonstrated that SERCA is differentially regulated through redox signaling as oxidants at low concentrations stimulate SERCA function (Adachi, 2010; Adachi et al., 2004; Cohen et al., 1999; Lancel et al., 2009; Smith et al., 2015; Tong et al., 2008; Tong, Evangelista, & Cohen, 2010; Trepakova, Cohen, & Bolotina, 1999; Tupling et al., 2007; Ying et al., 2007), whereas oxidants at high concentrations impair SERCA function (Adachi, 2010; Adachi et al., 2004; Cohen & Adachi, 2006; Gutiérrez-Martín, Martín-Romero, Iñesta-Vaquera, Gutiérrez-Merino, & Henao, 2004; Klebl, Ayoub, & Pette, 1998; Knyushko, Sharov, Williams, Schöneich, & Bigelow, 2005; Kuster et al., 2010; Lancel et al., 2010; Senisterra et al., 1997; Viner, Ferrington, Hühmer, Bigelow, & Schöneich, 1996; Viner, Williams, & Schöneich, 1999; Xu, Zweier, & Becker, 1997). One amino acid, Cys⁶⁷⁴, has been identified as an important redox-sensitive target that may function as SERCA's biological-switch, whereby reversible oxidation of Cys⁶⁷⁴ increases SERCA activity and irreversible oxidation impairs SERCA activity (Adachi, 2010; Adachi et al., 2004; Cohen et al., 1999; Lancel et al., 2009; Smith et al., 2015; Tong et al., 2008; Tong et al., 2010; Trepakova et al., 1999; Tupling et al., 2007; Ying et al., 2007). Additionally, lysine (Lys), tyrosine (Tyr), methionine (Met), and histidine (His) residues have also

been illustrated as oxidative sensitive targets; whereby, oxidation of these residues impairs SERCA function (Ahsan, 2013; Schöneich & Sharov, 2006; Stadtman & Levine, 2003; Tien, Berlett, Levine, Chock, & Stadtman, 1999).

SERCA impairment is often associated with oxidative damage to its cytoplasmic domains. A significant portion of SERCA's readily oxidized Cys residues, including Cys⁶⁷⁴, are cytoplasmic and freely accessible to water-soluble oxidants (Ariki & Shamoo, 1983; Senisterra et al., 1997). In addition to the irreversible oxidation of Cys⁶⁷⁴, oxidative damage to the nucleotide-binding domain (N-domain) also results in SERCA inactivation (Fu & Tupling, 2009; Luckin, Favero, & Klug, 1991; Matsushita & Pette, 1992; Tupling et al., 2004; Tupling, Green, Senisterra, Lepock, & McKee, 2001). In fact, when SERCA is exposed to oxidative stress, preservation of the structural integrity of the N-domain, as measured through fluorescein isothiocyanate (FITC) binding assays, is correlated with preserved SERCA function (Fu and Tupling, 2009; Tupling et al., 2004). FITC is a fluorescent probe that covalently labels Lys⁵¹⁵ (SERCA1a) (Pick & Karlsh, 1980) or Lys⁵¹⁴ (SERCA2a) (Fu & Tupling, 2009) located close to the ATP-binding site (Toyoshima et al., 2000). Therefore, oxidative damage to SERCA through irreversible

oxidation and/or through structural alterations of the pump results in SERCA impairment, as illustrated in myocardial ischemia (Temsah et al., 1999; Tupling et al., 2001), cardiovascular disease (Hobai et al., 2013; Knyushko et al., 2005), heat stress (Fu & Tupling, 2009; Schertzer, Green, & Tupling, 2002; Tupling et al., 2004), myopathies (Fajardo et al., 2015; Gehrig et al., 2012), and atherosclerosis (Adachi et al., 2004; Cohen & Adachi, 2006).

Although the SERCA isoforms have a high degree of sequence homology, it appears that distinctive protein modifications occur in different isoforms in response to oxidative stress (Barnes, Samson, & Grover, 2000; Grover, Kwan, & Samson, 2003; Grover, Samson, & Misquitta, 1997; Viner et al., 1996). Interestingly, there is evidence that SERCA2 is more susceptible to oxidation than other SERCA isoforms. It has been demonstrated that *in vitro* exposure of SR vesicles to peroxynitrite (ONOO⁻) yielded selective oxidation of SERCA2a in the presence of both SERCA2a and SERCA1a isoforms, even with excess SERCA1a protein (Viner et al., 1996). Additionally, several studies have demonstrated that SERCA3 is more resistant to oxidants than the SERCA2b isoform (Grover et al., 2003; Grover et al., 1997). A potential explanation for the increased sensitivity of SERCA2 towards oxidative

modifications when compared to the other SERCA isoforms is that with 26 Cys residues, SERCA2 has the highest number of the readily oxidized sulfonyl groups when compared to the other SERCA isoforms (Menguy et al., 1998; Senisterra et al., 1997); thereby, potentially providing SERCA2 with increased sensitivity towards oxidative regulation.

4-hydroxy-2-nonenal: A major product of lipid-peroxidation

In addition to oxidizing proteins, free-radicals readily oxidize polyunsaturated fatty acids (PUFAs). The mechanism of free-radical mediated lipid peroxidation (oxidation of lipids) consists of three distinct phases: initiation, propagation and termination (Porter, Caldwell, & Mills, 1995) (Figure 1). During the initiation phase, lipid peroxidation begins when free-radicals oxidize PUFAs, resulting in the formation of lipid radicals. PUFAs are particularly vulnerable to oxidation because they contain carbon-carbon double bonds with allylic hydrogens that are easily oxidized, resulting in the formation of resonance-stabilized lipid radicals (Ayala, Munoz, & Arguelles, 2014; Porter et al., 1995). Subsequently, the propagation of lipid peroxidation involves these newly generated lipid radicals reacting with oxygen to form lipid peroxy radicals (Ayala et al., 2014). Lipid peroxy radicals are extremely

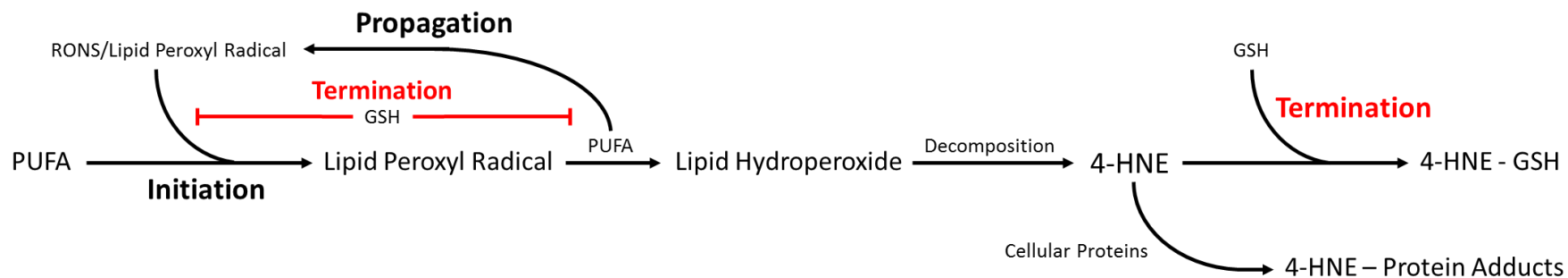


Figure 1: Simplified schematic illustrating the mechanism of non-enzymatic, free-radical mediated 4-HNE production and protein modification.

unstable and further react with other PUFAs, producing a lipid hydroperoxide product and a new lipid radical that further propagates the lipid peroxidation cascade (Ayala et al., 2014). The termination of lipid peroxidation occurs through naturally occurring antioxidant systems that reduce lipid radicals and stop the propagation of lipid peroxidation, the most notable with respect to 4-HNE is the cytosolic antioxidant glutathione (GSH) (Sinha-Hikim et al., 2011).

The lipid hydroperoxides that are generated as a result of lipid peroxidation are highly unstable and immediately decompose into secondary aldehydic products. The major secondary aldehydic products produced from lipid hydroperoxides are 4-hydroxy-2-nonenal (4-HNE), malondialdehyde (MDA), and acrolein (ACR) which are all considered to be α,β -unsaturated hydroxyalkenals (Ayala et al., 2014). Secondary aldehydic products are considered to be second messengers of free-radicals because they can bind to biomolecules and propagate free-radical signaling (Ayala et al., 2014). Notably, out of the three major end products, pioneering work in the 1980s by H. Esterbauer and colleagues has demonstrated that 4-HNE is produced in abundance and is the second most reactive product out of the three (Benedetti, Comporti, &

Esterbauer, 1980; Esterbauer, 1993; Esterbauer, Wäg, & Puhl, 1993; Schauenstein & Esterbauer, 1978).

4-HNE can functionally modify transmembrane proteins

4-HNE was first discovered in 1967 (Schauenstein, 1967) and it is produced from the oxidation of long chain (≥ 18 carbons in length), omega-6 (ω -6), polyunsaturated fatty acids (PUFAs), more specifically, arachidonic acid (AA) 20:4(ω -6), linoleic acid (LA) 18:2(ω -6) and γ -linolenic acid (GLA) 18:3(ω -6) (Ayala et al., 2014; Poli, Biasi, & Leonarduzzi, 2008; Poli, Schaur, Siems, & Leonarduzzi, 2008; Pryor & Porter, 1990; Riahi, Cohen, Shamni, & Sasson, 2010; Schneider, Tallman, Porter, & Brash, 2001; Uchida, 2003; Uchida, Szweda, Chae, & Stadtman, 1993). 4-HNE has a unique molecular structure containing a carbon-carbon double bond, a hydroxyl group and an aldehydic functional group, which all collectively contribute to its strong electrophilic characteristics (Ayala et al., 2014). Due to its strong electrophilic characteristics, 4-HNE rapidly reacts with nucleophilic regions of proteins through direct protein adduction (Petersen & Doorn, 2004; Stadtman & Levine, 2003). In an excellent review, Madian and Regnier describe how proteins can be oxidized in more than 35 different ways,

and that these post-translational protein modifications can be divided into three specific categories; protein adduction by the end products of lipid peroxidation was identified as one of the three classifications (Madian & Regnier, 2010). Interestingly, similar to free-radicals, 4-HNE rapidly oxidizes Cys, His, and Lys residues (Petersen & Doorn, 2004; Schaur, 2003).

The electrophilic and lipophilic characteristics of 4-HNE places membranous proteins vulnerable to 4-HNE adduction, particularly because of their close proximity to the site of 4-HNE production. Interestingly, 4-HNE adduction onto the Na⁺/K⁺ pump leads to a dose-dependent inactivation of the enzyme (Morel, Tallineau, Pontcharraud, Piriou, & Huguet, 1998; Siems, Hapner, & Kuijk, 1996). The Na⁺/K⁺ pump possesses a high number of Cys residues (Siems et al., 1996) and Morel and colleagues have demonstrated that 4-HNE treatment significantly decreases the Cys content of the Na⁺/K⁺ pump, resulting in the loss of free -SH groups and -SH group functionality (Morel et al., 1998). Similarly, 4-HNE has been shown to alter SERCA2a Ca²⁺-uptake in a biphasic manner, with low concentrations potentially increasing SERCA2a activity and higher concentrations inhibiting activity (Tian et al., 2014). To date, no study has investigated the effects of 4-HNE on

SERCA structure or if SERCA isoforms are differentially affected by 4-HNE.

Purpose:

The objectives of this thesis are to determine the effects of 4-HNE on the structure and function of SERCA1a and SERCA2a pumps.

Hypothesis:

- 1) 4-HNE will dose-dependently impair SERCA pumps upon co-incubation. This will be demonstrated by a reduction in maximal Ca^{2+} -dependent SERCA activity of both SERCA1a (fast skeletal muscle) and SERCA2a (left ventricle).
- 2) 4-HNE will dose-dependently adduct onto the SERCA1a and SERCA2a pump isoforms as measured through Western blotting.
- 3) 4-HNE will dose-dependently increase SERCA aggregation as illustrated through increased higher molecular weight bands (>100kDa) and a reduction in SERCA monomer when Western blotting for SERCA1a and SERCA2a after 4-HNE treatment.
- 4) 4-HNE treatment will have greater effects on SERCA2a when compared to SERCA1a, as illustrated through the above measures.

Methods

Animals

Adult (> 10 weeks) C57BL/6J mice were used in this study. These animals had access to water and laboratory chow ad libitum and were housed in a temperature controlled room on a reverse light/dark cycle until tissue collection. Immediately after culling the white portions of the gastrocnemius (WG) and whole left ventricle (LV) tissues were excised, homogenized in PMSF buffer (250mM sucrose, 5mM HEPES, 0.2mM PMSF, and 0.2% NaN₃), aliquoted, immediately frozen in liquid nitrogen, and subsequently stored at -80 °C until experimental use. The experimental protocols were reviewed and approved by the University of Waterloo Animal Care Committee.

4-HNE Treatment Protocol

To investigate the effects of 4-HNE on the structure and function of the SERCA pump, WG and LV whole tissue homogenates were incubated with different concentrations of 4-HNE. Initially, 4-HNE (sc-202019, Santa Cruz Biotechnology) was reconstituted in 2mL of ethanol to achieve a concentration of 0.5mg/mL. Upon use, 4-HNE was added into disposable glass culture tubes (14-961-25, Fisher Scientific) and dried

with gentle heat and nitrogen gas to evaporate the ethanol solution. The respective treatment concentrations (0mM, 0.5mM, 1mM, 2.5mM, 5mM, and 10mM) were achieved after the addition of different volumes of 4-HNE, evaporation of ethanol, and subsequent addition of 2.5uL of dimethyl sulfoxide (DMSO) (276855-100mL, Sigma-Aldrich) and 10uL of muscle homogenate. The samples containing the 4-HNE, DMSO and muscle homogenates were treated at room temperature (24 °C) for 1 hour and gently vortexed at time-points: 0, 30 and 60 minutes during the incubation protocol. After treatment, the samples were either assayed for maximal SERCA activity or further processed for assessment of post-translational structural modifications of the SERCA isoforms as described below.

Maximal Ca²⁺-dependent ATPase activity

Measurements of Ca²⁺-dependent SERCA activity were made at 37 °C using a spectrophotometric assay developed by (Simonides & Hardeveld, 1990) that has been adapted to a 96-well plate reader (Duhamel et al., 2007). To investigate the effects of 4-HNE on maximal SERCA activity, both WG and LV whole tissue homogenates were treated with 4-HNE at the respective treatment concentrations. After

incubation, 7.5uL of each of the treated samples were then added to 5mL of SR Ca²⁺ ATPase buffer (pH = 7, 200mM KCl, 20mM HEPES, 10mM NaN₃, 1mM EGTA, 15mM MgCl₂, 5mM ATP, and 10mM phosphoenolpyruvate (PEP)) and subsequently, 18U/mL of pyruvate kinase (PK) (10-128-163-001, Roche), 18U/mL of lactate dehydrogenase (LDH) (L2625-25KU, Sigma Life Sciences), and 1 uM calcium ionophore A23187 (C-7522, Sigma-Aldrich) were combined in a test tube on ice. The cocktail was then vortexed and subdivided (300uL) into 16 microcentrifuge tubes with varying concentrations of CaCl₂, allowing for the measurement of Ca²⁺-dependent maximal SERCA activity. The free Ca²⁺ corresponding to each CaCl₂ addition was assessed separately by use of single-wavelength spectrofluorometry and the Ca²⁺-fluorescent dye Indo-1 (I-1203, Life Technologies). The range of Ca²⁺ additions translates into a *p*Ca range of ~ 7.0 - 5.0. Next, each of the sixteen 300uL subdivisions were loaded in duplicate (100uL) onto a clear round-bottom 96-well plate. The final step was the addition of 1uL of 1.7% (w/v) NADH (13348539, Roche) to each well. The ATPase activity is represented by the decrease in NADH (606-68-8, Sigma-Aldrich) absorbance at 340nm (SPECTRAMax Plus, Molecular Devices). To distinguish Ca²⁺-ATPase activity from background ATPase activity,

240uM of the SERCA specific inhibitor cyclopiazonic acid (CPA) (111258-00-5, Sigma-Aldrich) was used (Seidler, Jona, Vegh, & Martonosi, 1989). The difference between the total activity and the basal activity (activity in the presence of CPA) represented the SERCA specific Ca²⁺-ATPase activity. The reaction proceeded for 30 minutes at 37 °C and the rate of decline in NADH was kinetically recorded beginning at minute 10 of the trial. All values were normalized to total protein concentration as determined through the bicinchoninic acid (BCA) assay. The V_{Max} was taken as the peak rate of activity, which typically occurred between a pCa of 5.5 - 5.0.

Isolation of SERCA1a and SERCA2a via immunoprecipitation (IP)

In order to accurately determine SERCA-specific (SERCA1a and SERCA2a) 4-HNE adduction, nitrotyrosine formation, glutathionylation and FITC binding capacity, SERCA1a and SERCA2a were immunoprecipitated from the treated WG and LV homogenates, respectively. This yielded SERCA specific measures and removed other possible confounding proteins, as the SERCA pump and Na⁺/K⁺ pump are both members of the P-type transporter family and are of similar size (~100kDa) and 3-D structure (Morth et al., 2007; Toyoshima 2000). First,

SERCA1a or SERCA2a primary antibodies were coupled with anti-IgG coupled agarose beads. Initially, 75 μ L of the IgG coupled agarose beads were washed with phosphate buffered saline (PBS) (pH=7.4, 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, and 1.42mM NaH₂PO₄) and the antibodies were subsequently coupled to the agarose bead by adding 0.5 μ L or 1 μ L of SERCA1a or SERCA2a primary antibody, respectively. After the addition of the respective antibodies the samples were brought to a final volume of 50 μ L with PBS and subsequently incubated for 2 hours. After primary incubation, the beads were washed with blocking solution (pH=7.4 1% BSA in PBS) by incubating for 15 minutes on ice with sporadic shaking. After blocking the beads were washed with PBS solution and the antibody coupled agarose beads were then incubated with 7.5 μ L of the treated sample homogenates with 192.5 μ L of Co-IP buffer (pH = 7.4, 150mM NaCl, 20mM HEPES, 1mM EDTA, 10mM MgCl₂, and 0.5% Tween (v/v)). The cocktail containing the treated homogenate and antibody coupled agarose beads were then subsequently incubated for 1 hour on an orbital shaker in the fridge at 4 °C. After incubation, the samples were centrifuged and then washed with Co-IP buffer three times and one last time with 0.5mL of wash buffer (pH=7.2, 150mM NaCl, 25mM Tris base), the supernatant was

discarded. The immunoprecipitated SERCA1a and SERCA2a protein were then eluted from the cocktail by adding 36 μ L of low pH buffer (pH= 2.8 100mM glycine) and incubated on ice for 15 minutes. The eluted material (containing SERCA1a or SERCA2a as well as the eluted antibody) was collected and neutralized with neutralization buffer (pH = 9.0 1M Tris Base). The samples were then either Western blotted or treated with FITC.

Western Blotting of whole homogenate and IP samples

To investigate the effects of 4-HNE on SERCA structure, which may contribute to changes in SERCA function, both WG and LV whole tissue homogenates were subjected to Western blotting following 4-HNE treatment (0, 0.25, 1.0, 2.5, 5.0, and 10mM). Samples were either mixed with 12.5 μ L of sample reducing buffer (pH = 6.8, 0.1M Tris-HCl, 45% glycerol (v/v), 1mM SDS, 0.037mM bromophenol blue, and 12.5% (v/v) B-mercaptoethanol) to detect 4-HNE protein adduction and nitrotyrosine formation or with sample non-reducing buffer (pH = 6.8, 0.1M Tris-HCl, 45% glycerol (v/v), 1mM SDS, and 0.037mM Bromophenol blue) to detect protein glutathionylation and SERCA aggregation. A total of 4 μ L of the treated homogenates (~ 35-50 μ g) were loaded onto 7.5% polyacrylamide

gels and separated using standard SDS-PAGE protocols (Laemmli, 1970). A molecular weight marker (Precision Plus Protein Western C Standards; 161-0376, Bio-Rad) was added to identify the molecular weights of the separated proteins. The samples were separated using the Bio-Rad Power-Pak 200 set at an initial voltage of 100 V for 10-15 minutes and then at 120 V for 55-60 minutes for a total time of 70 minutes. After electrophoresis, the gels were then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad) using wet-transfer techniques and subsequently blocked for 1 hour at room temperature in 5% skim-milk with Tris-buffered saline plus Tween (TBS-T; Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) on a tilt-table. After blocking, antibodies for 4-HNE (MAB3249, Research and Development), 3-nitrotyrosine (189542, Cayman Chemicals), glutathionylation (101-A-250, Virogen), SERCA1a (kindly donated by Dr. Maclennan), and SERCA2a (MA3-919, Affinity BioReagents) were applied for 1 hour at room temperature on a rocker table (See Appendix, Table A1). After primary incubation, the membranes were washed with TBS-T for a total of 15 minutes (3 washes at 5 minutes/wash) and probed for 1 hour with horse-radish peroxidase (HRP) conjugated anti-mouse secondary antibody (See Appendix, Table A1). After incubation with secondary antibody, the membranes were

washed for a total of 15 minutes (3 washes at 5 minutes/wash) with TBS-T and the images were visualized using ECL Western blotting substrate kit (K820-500-1, BioVision) and a bio-imaging system. Images were captured and analyzed using the software GeneSnap and GeneTools respectively (Syngene). For whole homogenate Western blots, the signals in all of the lanes were normalized to total protein as detected through membrane Ponceau staining and subsequently normalized to control lanes to yield relative changes respective to total protein load and control condition. For immunoprecipitation Western blots, the signals in all of the lanes were normalized to their respective SERCA content and subsequently normalized to control lanes to yield relative changes respective to SERCA load and control condition.

Determination of FITC binding to SERCA1a or SERCA2a

FITC binding to SERCA was assessed in immunoprecipitated samples following neutralization. After neutralization, the immunoprecipitated samples were combined with 80 μ L of FITC binding buffer (pH = 8.8, 50mM Tris-HCl, 250mM sucrose, 0.1mM CaCl₂, 5mM MgCl₂, 20uM FITC and protease inhibitors) and incubated at room temperature in the dark for 1 hour. To stop the reaction, reducing buffer

was added to each sample. Next, 10 μ L of each sample were loaded onto a 7.5% polyacrylamide gel and separated by SDS-PAGE. The membranes were probed with anti-flouroscein/Oregon Green monoclonal antibody (4-4-20, Invitrogen) (See Appendix, Table A1) in milk in TBS-T. Lastly the membranes were probed with anti-mouse secondary antibody (See Appendix, Table A1) and the images were developed using GeneSnap (Syngene). The optical density for each of the treatment concentrations was normalized to its respective control sample.

Data Analysis

All data are expressed as means \pm standard error of the mean (S.E.). Statistical significance was set to $P < 0.05$. The statistical analysis was carried out using either a one-way repeated measures ANOVA or a two-way ANOVA where appropriate. Post-hoc analysis was carried out using Tukey's test for significance.

Results

4-HNE adduction and Ca²⁺-ATPase activities

Western blots demonstrated that 4-HNE treatment resulted in a significant increase in total protein 4-HNE adduction in both WG and LV tissues (Figure 2). In WG, treatment concentrations of 5.0 and 10.0mM caused greater 4-HNE adduction compared with CTL, 1.0mM, and 2.5mM conditions (Fig. 2A and E). In LV, 4-HNE protein adduction was increased following treatment with 5.0mM 4-HNE and further increased with 10.0mM treatment (Fig. 2B and F).

Immunoprecipitation of SERCA1a and SERCA2a from WG and LV tissues, respectively, demonstrated increased 4-HNE adduction to SERCA1a (Fig. 3A and E) with 10mM 4-HNE treatment. Results illustrating an approximate 147% increase in 4-HNE – SERCA1a adduction when compared to CTL. In contrast, there were no observable changes in the SERCA2a isoform (Fig. 3B and F).

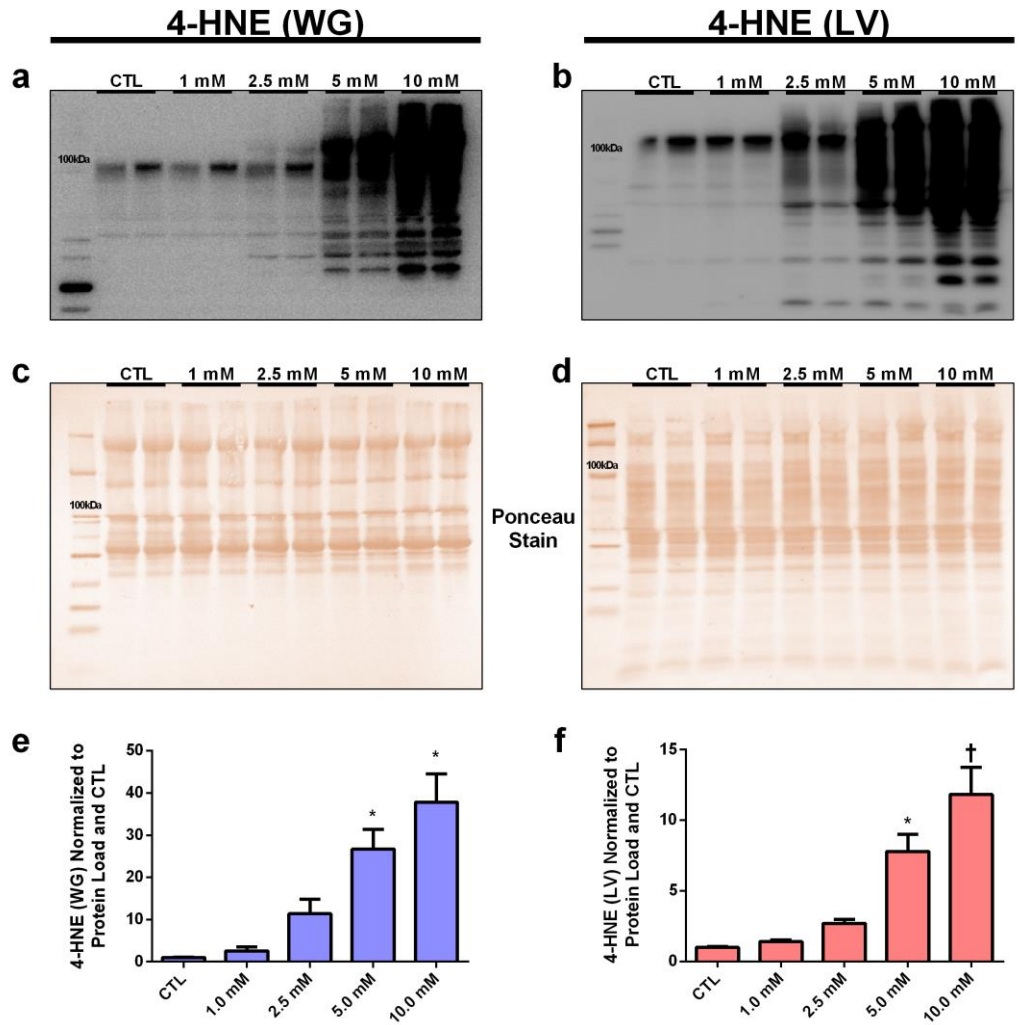


Figure 2: 4-HNE Western blots for WG and LV tissue.

(A) A representative Western blot of 4-HNE adduction to WG homogenate. **(B)** A representative Western blot of 4-HNE adduction to LV homogenate. **(C)** A Ponceau stain of the representative (WG) Western blot used for normalization to total protein load. **(D)** A Ponceau stain of the representative (LV) Western blot used for normalization to total protein load. **(E)** Bar graph of 4-HNE adduction to WG homogenate. **(F)** Bar graph of 4-HNE adduction to LV homogenate. All data are means \pm S. E. (WG: n = 9; LV: n = 18)

*, $p < 0.05$ versus CTL, 1.0mM and 2.5mM treatment groups; †, $p < 0.05$ versus all treatment groups.

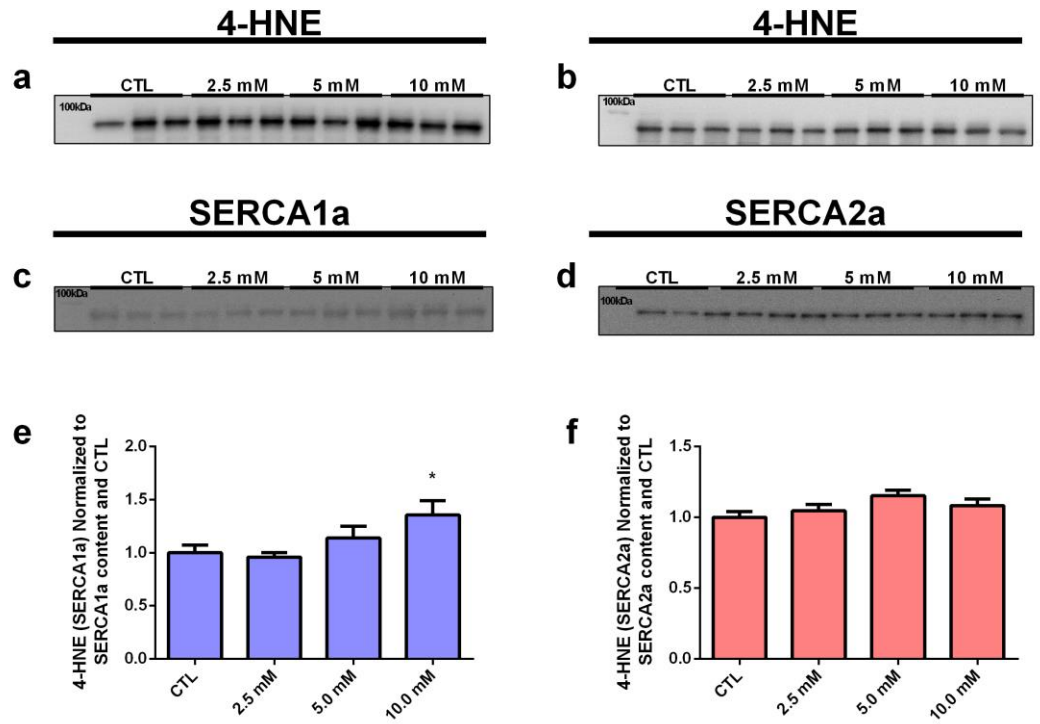
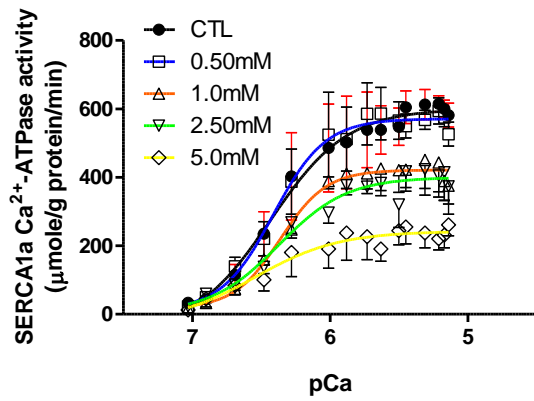


Figure 3: 4-HNE Western blots for Immunoprecipitated SERCA1a and SERCA2a.

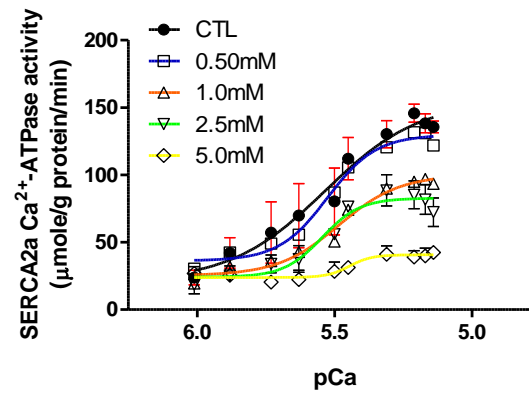
(A) A representative Western blot of SERCA1a 4-HNE protein adduction. **(B)** A representative Western blot of SERCA2a 4-HNE protein adduction. **(C)** A Western blot of the immunoprecipitated SERCA1a used to normalize for SERCA1a protein load. **(D)** A Western blot of the immunoprecipitated SERCA2a used to normalize for SERCA2a protein load. **(E)** Bar graph of SERCA1a 4-HNE protein adduction **(F)** Bar graph of SERCA2a 4-HNE protein adduction. All data are means \pm S. E. (WG: n = 7; LV: n = 9).
 *, $p < 0.05$ versus CTL group.

To determine if 4-HNE functionally impairs SERCA activity, WG and LV homogenates were assayed for maximal Ca^{2+} -dependent ATPase activities following 4-HNE treatment. The dose-dependent effects of 4-HNE on SERCA function are shown in Figure 4 and Table 1. In WG, there was a trend ($p = 0.06$) for a lower (~66% relative to CTL) maximal SERCA activity following 4-HNE treatment for both 1.0 and 2.5mM concentrations; however, 5.0mM 4-HNE treatment caused a significant 57% decrease in maximal Ca^{2+} -dependent ATPase activity when compared with CTL (Fig. 4A and C; Table 1). 4-HNE treatment in LV caused a dose-dependent decrease in maximal Ca^{2+} -dependent SERCA2a activity beginning at 1.0mM 4-HNE treatment (Fig. 4B and C; Table 1). There were no effects of 4-HNE treatment on the apparent Ca^{2+} -affinities of SERCA1a and SERCA2a between all treatment conditions (Table 1). There were also no differences in the Hill slope (n_H) between treatment conditions in either WG or LV (Table 1).

4.A



4.B



4.C

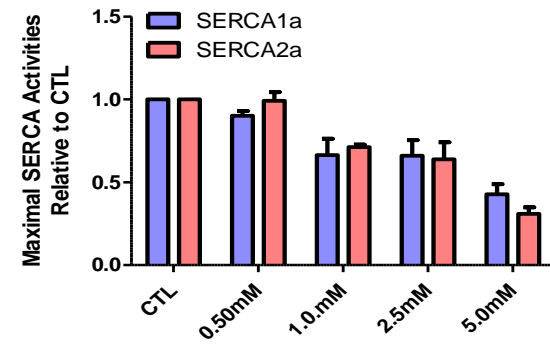


Figure 4: Ca²⁺-dependent SERCA activity for SERCA1a and SERCA2a.

(A) pCa curve illustrating Ca²⁺-dependent maximal SERCA1a activity across 4-HNE treatment concentrations **(B)** pCa curve illustrating Ca²⁺-dependent maximal SERCA2a activity across 4-HNE treatment concentrations. **(C)** Bar graph comparing 4-HNE inhibition of both SERCA isoforms relative to their respective CTLs. All data are means ± S. E. (WG: n = 6; LV: n = 4).

Table 1: Effects of 4-HNE on SERCA enzyme kinetics

WG and LV homogenate was incubated with 4-HNE and maximal Ca²⁺-dependent SERCA2a activity was subsequently assessed. All data are means ± S. E. (WG: n = 6; LV: n = 4).

	<i>4-hydroxy-2-nonenal Treatment Concentration</i>									
	CTL		0.5 mM		1.0 mM		2.5 mM		5.0 mM	
	WG	LV	WG	LV	WG	LV	WG	LV	WG	LV
V_{Max}	755.57 ± 29.4	146.6 ± 6.10	685.36 ± 46.5	145.05 ± 5.23	501.45 ± 74.7	104.28 ± 4.22 *	498.2 ± 71.29	93.34 ± 8.26 *	327.21 ± 52.44 *	45.53 ± 3.98 †
K_{Ca}	6.32 ± 0.10	6.02 ± 0.06	6.41 ± 0.09	5.98 ± 0.08	6.52 ± 0.06	5.99 ± 0.04	6.49 ± 0.07	6.01 ± 0.02	6.38 ± 0.10	6.14 ± 0.09
n_H	3.45 ± 0.51	3.50 ± 1.12	3.18 ± 0.31	2.80 ± 0.32	4.18 ± 0.55	2.23 ± 0.20	4.46 ± 1.02	3.70 ± 0.81	2.64 ± 0.39	2.12 ± 0.55

*, p < 0.05 compared within their respective CTL & 0.5mM treatment groups;

†, p < 0.05 compared within all their respective treatment groups.

(WG): CTL versus 1.0mM p=0.06;

(WG): CTL versus 2.5mM p=0.06.

Western blot results

To determine the mechanism(s) responsible for 4-HNE mediated impairment of maximal Ca^{2+} -dependent SERCA activity, Western blotting was used to measure nitrotyrosine formation, glutathionylation, and SERCA aggregation in whole muscle homogenates from WG and LV tissue after 4-HNE treatment. There were no detectable increases in total nitrotyrosine formation (Figure 5) or glutathionylation (Figure 6) in either WG or LV. Western blotting for SERCA1a aggregation in WG revealed a significant reduction in SERCA1a monomer levels following 2.5mM, 5.0mM and 10mM 4-HNE treatment when compared to CTL and 1.0mM 4-HNE treatment groups (Fig. 7A and E). Corresponding with reduced SERCA1a monomer levels, compared with CTL, higher molecular weight SERCA1a aggregates were increased by ~400% and ~800% in samples that were treated with 5.0 and 10.0mM 4-HNE, respectively, but only the difference between CTL and 10.0mM 4-HNE was significant. (Fig. 7A and G). Western blotting for SERCA2a aggregation revealed a significant decrease in SERCA2a monomer levels in samples that were treated with 5.0mM and 10.0mM 4-HNE when compared to CTL, 1.0mM, and 2.5mM 4-HNE treatment groups (Fig. 7B and F). Western blotting for higher molecular weight SERCA2a

aggregates revealed higher levels in 2.5mM, 5.0mM and 10.0mM 4-HNE treatment groups when compared with CTL (Fig. 7B and H).

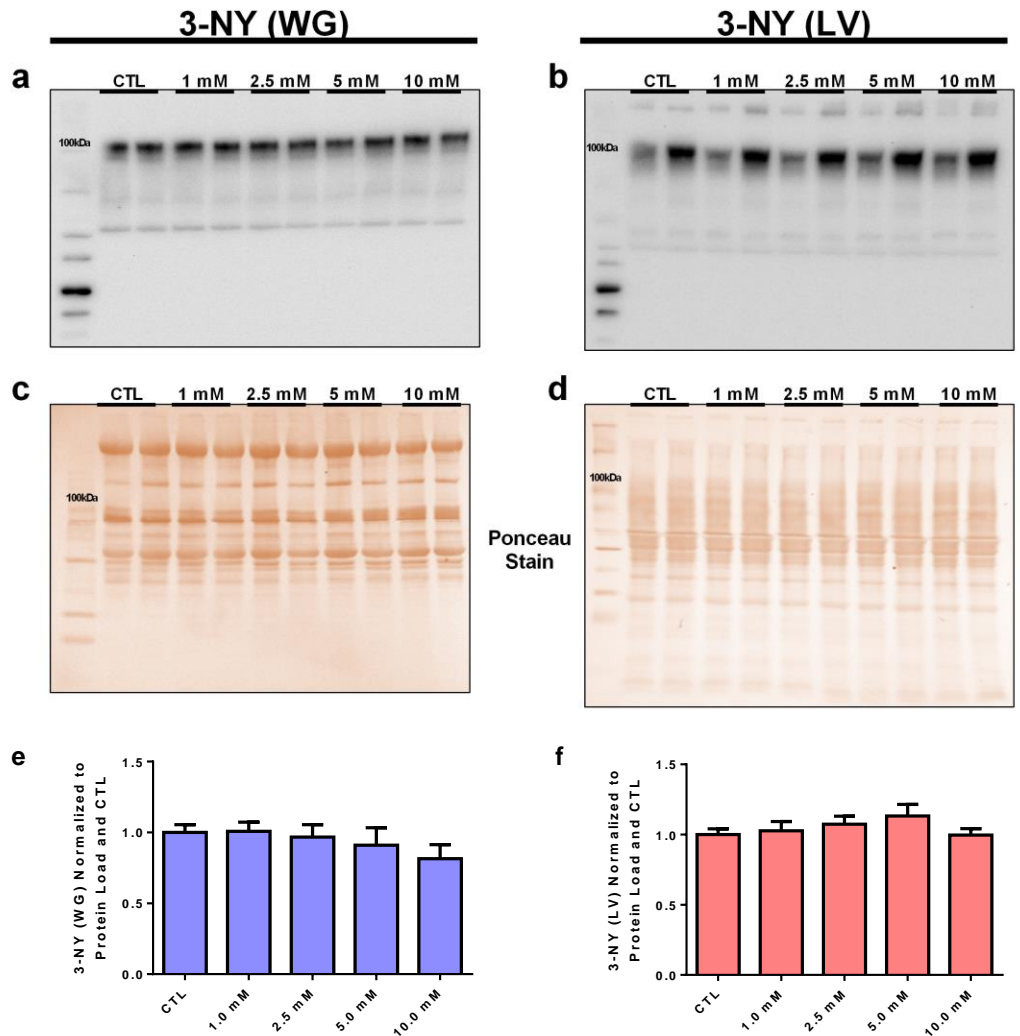


Figure 5: 3-Nitrotyrosine formation Western blots for WG and LV tissue.

(A) A representative Western blot of 3-nitrotyrosine formation to WG homogenate. **(B)** A representative Western blot of 3-nitrotyrosine formation to LV homogenate. **(C)** A Ponceau stain of the representative (WG) Western blot used for normalization to total protein load. **(D)** A Ponceau stain of the representative (LV) Western blot used for normalization to total protein load. **(E)** Bar graph of 3-nitrotyrosine formation to WG homogenate. **(F)** Bar graph of 3-nitrotyrosine formation to LV homogenate. All data are means \pm S. E. (WG: n = 8; LV: n = 6).

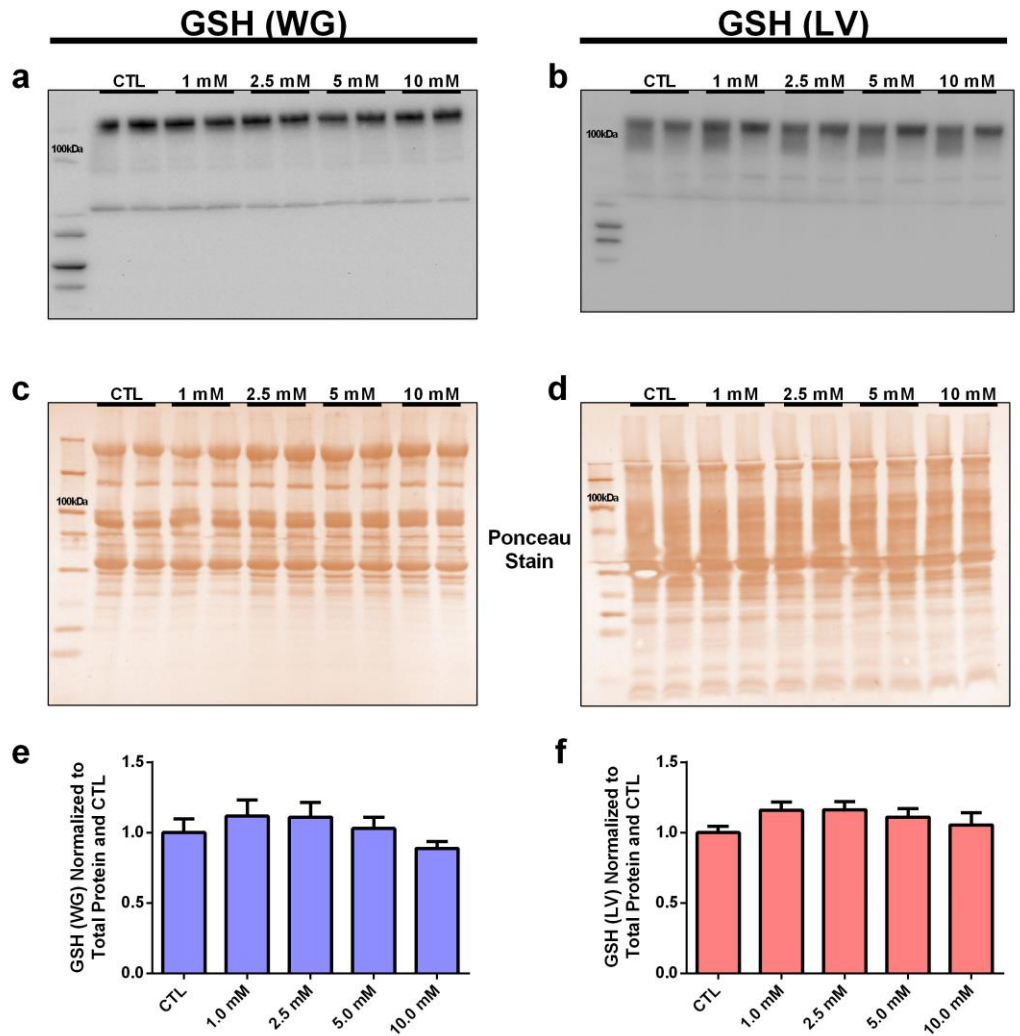


Figure 6: Glutathionylation Western blots for WG and LV tissue.

(A) A representative Western blot of protein glutathionylation to WG homogenate. **(B)** A representative Western blot of protein glutathionylation to LV homogenate. **(C)** A Ponceau stain of the representative (WG) Western blot used for normalization to total protein load. **(D)** A Ponceau stain of the representative (LV) Western blot used for normalization to total protein load. **(E)** Bar graph of glutathionylation to WG homogenate. **(F)** Bar graph of glutathionylation to LV homogenate. All data are means \pm S. E. (WG: n = 8; LV: n = 12).

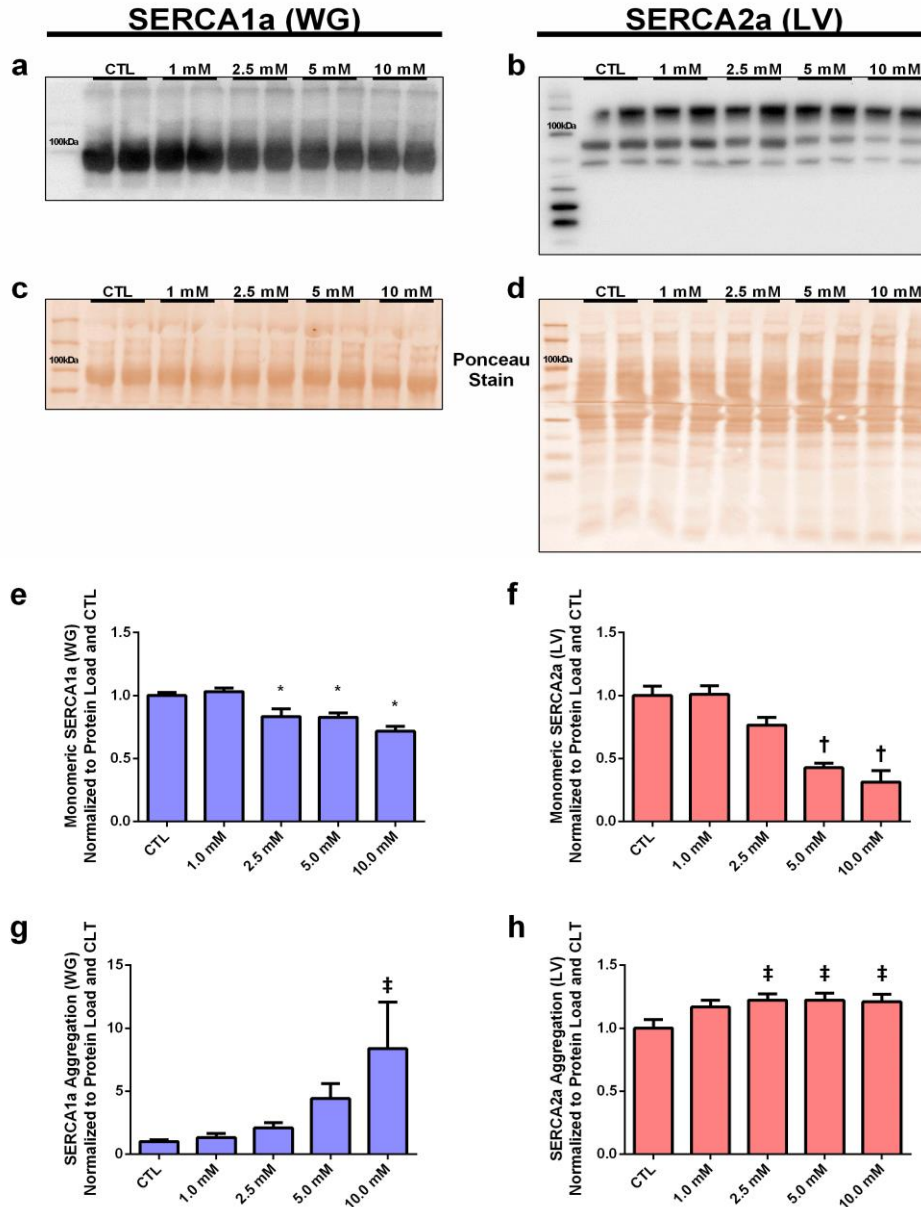


Figure 7: SERCA aggregation Western blots for WG and LV tissue.

(A) A representative Western blot of SERCA1a in WG homogenate. **(B)** A representative Western blot of SERCA2a in LV homogenate. **(C)** A Ponceau stain of the representative (WG) Western blot used for normalization to total protein load. **(D)** A Ponceau stain of the representative (LV) Western blot used for normalization to total protein load. **(E)** Bar graph of SERCA1a monomer. **(F)** Bar graph of SERCA2a monomer. **(G)** Bar graph of SERCA1a aggregates (>100kDa). **(H)** Bar graph of SERCA2a aggregates (>100kDa). All data are means \pm S. E. (WG: n = 6; LV: n = 8).

*, p < 0.05 versus CTL and 1.0 mM treatment groups; †, p < 0.05 versus CTL, 1.0 mM and 2.5 mM treatment groups; ‡, p < 0.05 versus CTL treatment group.

To determine if 4-HNE causes SERCA specific post-translational modifications, SERCA1a and SERCA2a were immunoprecipitated from WG and LV tissues, respectively. Western blotting of immunoprecipitated SERCA1a and SERCA2a revealed no increases in nitrotyrosine formation of either SERCA isoform (SERCA1a: Fig. 8C and I; SERCA2a Fig. 8D and J). Additionally, there were no detected increases in SERCA specific glutathionylation in either isoform (SERCA1a: Fig. 8E and K; SERCA2a: Fig. 8F and L). Interestingly, FITC binding assays demonstrated a reduction in FITC binding to SERCA1a with 5.0mM and 10.0mM 4-HNE treatment (Fig. 8G and M). There were no observed changes in FITC binding to the SERCA2a isoform with 4-HNE treatment (Fig. 8H and N).

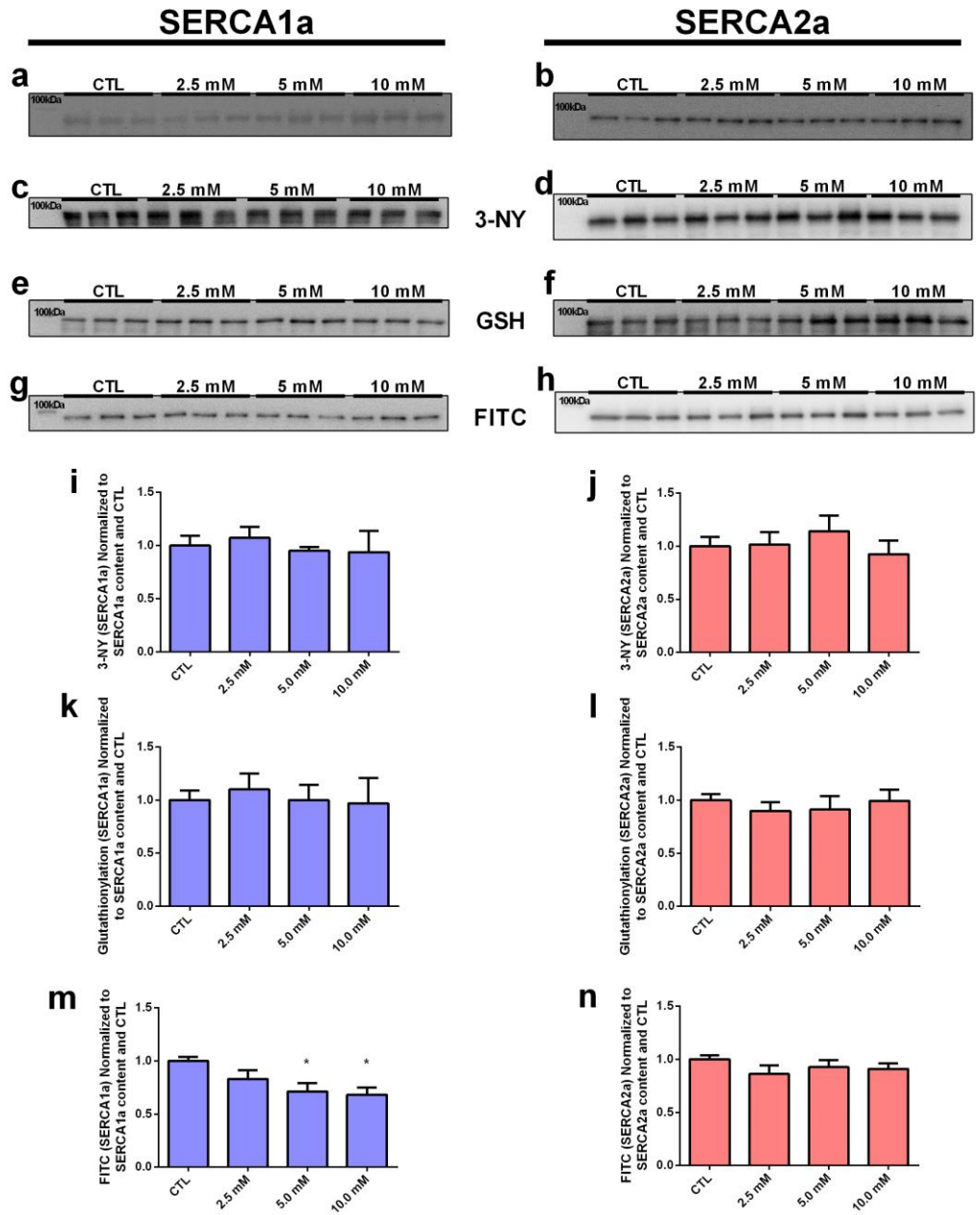


Figure 8: Western blots for Immunoprecipitated SERCA1a and SERCA2a.

(A) A Western blot of the immunoprecipitated SERCA1a used to normalize for SERCA1a protein load. (B) A Western blot of the immunoprecipitated SERCA2a used to normalize for SERCA2a protein load. (C) A representative Western blot of SERCA1a nitrotyrosine formation. (D) A representative Western blot of SERCA2a nitrotyrosine formation. (E) A representative Western blot of SERCA1a glutathionylation. (F) A representative Western blot of SERCA2a glutathionylation. (G) A representative Western blot of SERCA1a FITC binding. (H) A representative Western blot of SERCA2a FITC binding. (I) Bar graph of SERCA1a nitrotyrosine formation. (D) Bar graph of SERCA2a nitrotyrosine formation. Data are means \pm S. E. (WG: n = 6; LV: n = 6). (K) Bar graph of SERCA1a glutathionylation. (L) Bar graph of SERCA2a

glutathionylation. Data are means \pm S. E. (WG: n = 6; LV: n = 6). **(M)** Bar graph of SERCA1a FITC binding. **(D)** Bar graph of SERCA2a FITC binding. Data are means \pm S. E. (WG: n = 3; LV: n = 6). *, p < 0.05 versus CTL treatment group.

Discussion

This study utilized mouse WG and LV to investigate the effects of 4-HNE on SERCA1a and SERCA2a Ca²⁺-dependent maximal ATPase activities, respectively. Additionally, the structural integrity and post-translational modifications of SERCA were investigated to identify potential mechanisms of 4-HNE mediated SERCA impairment. To determine an appropriate treatment protocol the methods were originally adapted from Tian and colleagues (Tian et al., 2014). The treatment range of 4-HNE was determined from studies illustrating that concentrations of 5 - 10 mM were near maximal pathophysiological concentrations that 4-HNE would reach *in-vivo* (Esterbauer, Schaur, & Zollner, 1991; Smathers, Galligan, Stewart, & Petersen, 2011; Uchida, 2003). As hypothesized, 4-HNE incubation caused an increase in 4-HNE – protein adduction (Figure 2) and 4-HNE – SERCA1a adduction (Fig. 3A and E) in 4-HNE treated samples. These results are consistent with other studies reporting 4-HNE – protein adducts (Uchida, 2003; Uchida et al., 1993); however, purification of SERCA2a from LV treated homogenates revealed no significant increases in 4-HNE – SERCA2a adducts (Fig. 3B and F). This is the first study to-date demonstrating increased 4-HNE adduction to the SERCA pump; however, because only SERCA1a had

significantly increased 4-HNE adducts, these results reveal that 4-HNE potentially causes isoform specific changes upon co-incubation with SERCA.

As expected, 4-HNE treatment resulted in reductions of maximal SERCA1a and SERCA2a Ca²⁺-ATPase activities in WG and LV tissues, respectively (Figure 4; Table 1). In the only other study to date that examined the effects of 4-HNE on SERCA function, Tian and colleagues report similar findings, in that 4-HNE treatment dose-dependently reduced SR Ca⁺-uptake in re-suspended cellular vesicles from rat heart; however, they were unable to detect 4-HNE adducts onto the SERCA2a pump (Tian et al., 2014). Additionally, *in vitro* studies have examined the effects of 4-HNE on the structure and function of the Na⁺/K⁺ pump (Morel et al., 1998); the Na⁺/K⁺ and SERCA pumps are of similar size (~100kDa) and 3-D structure (Morth et al., 2007; Toyoshima, 2000). Similarly, Morel and colleagues demonstrate that 4-HNE treatment to rat synaptosomes results in a dose-dependent reduction of Na⁺/K⁺ pump activity (Morel et al., 1998). Therefore, the observed reductions in maximal SERCA1a and SERCA2a activity confirm our hypothesis and is in agreement with the current literature; whereby, 4-HNE is a bioactive molecule capable of inhibiting protein function.

Prior research has established that SERCA can be inactivated through nitrosylation, carbonylation, glutathionylation, and aggregation of the pump (Adachi et al., 2004; Fu & Tupling, 2009; Senisterra et al., 1997). 4-HNE treatment and the resulting impairments in SERCA function were independent of protein and SERCA specific nitrotyrosine formation (Fig. 5; Fig. 8I and J) and glutathionylation (Fig. 6; Fig. 8K and L); however, we did observe increased SERCA1a and SERCA2a aggregates following 4-HNE treatment (Figure 7). The increased SERCA aggregation is expected and in agreement with our hypothesis. 4-HNE is documented to cause structural destabilization and protein crosslinking leading to protein aggregation (Zarkovic, Cipak, Jaganjac, Borovic, & Zarkovic, 2013). Furthermore, 4-HNE is extremely reactive towards Cys residues which is the predominate mechanism by which SERCA is inactivated through free-radicals. Therefore, 4-HNE oxidizing Cys residues and causing SERCA aggregation could be the leading mechanism causing the observed 4-HNE induced SERCA inactivation.

Interestingly, there were isoform specific structural changes following 4-HNE treatment; SERCA1a had reductions in FITC binding (Fig. 8G and M). The observed reductions of FITC binding to SERCA1a reinforce the notion that 4-HNE induces structural destabilization of

proteins, as the reductions of FITC binding indicate structural destabilization of the SERCA1a nucleotide binding domain.

Interestingly, FITC binding to SERCA2a was preserved in 4-HNE treated homogenates which was unexpected. The preserved FITC binding to SERCA2a could potentially be explained by Hsp70. Hsp70 is a cytoprotective molecule that can bind to both SERCA1a and SERCA2a isoforms (Fu & Tupling, 2009; Tupling et al., 2004), preserving SERCA function in conditions of enhanced oxidative stress. Hsp70 is constitutively expressed in both skeletal and cardiac muscle but in skeletal muscle, its expression tends to coincide with oxidative type I/IIA muscle fibres (Locke, Noble, & Atkinson, 1991; Neuffer et al., 1996; Tarricone et al., 2008; Tupling, Bombardier, Stewart, Vigna, & Aquilino, 2007). Therefore, because mouse WG is predominantly fast-twitch type IIB (Bloemberg & Quadrilatero, 2012), the differences in FITC binding capacity could be attributed to Hsp70 expression; whereby, Hsp70 was able to preserve SERCA2a's N-domain through direct binding to the pump. In contrast, SERCA1a's N-domain was potentially destabilized because mouse WG does not endogenously express appreciable levels of Hsp70. Overall, it can be concluded that the structural destabilization of

SERCA1a's N-domain at least partially explains the inactivation of SERCA1a caused by 4-HNE treatment.

4-HNE treatment to WG and LV homogenates resulted in increased 4-HNE adduction to SERCA1a (Fig. 3A and E) but not to SERCA2a (Fig. 3B and F). These isoform specific changes can be explained by the cellular contents of reduced GSH in WG and LV tissue, respectively. The WG contains less reduced GSH than LV tissue (Lancel et al., 2009). Reduced GSH is the predominant mechanism that protects against 4-HNE stress as 4-HNE can directly adduct onto GSH producing 4-HNE – GSH conjugates, preventing 4-HNE adduction to other cellular proteins (Ålin, Danielson, & Mannervik, 1985; Sinha-Hikim et al., 2011; Yang, Sharma, Sharma, Awasthi, & Awasthi, 2003). This conclusion is supported by the observed ~40-fold increase in 4-HNE adduction to total protein in WG (Fig. 2A and E) versus the ~13-fold increase in LV (Fig. 2B and F) with 10mM 4-HNE treatment, potentially illustrating GSH's buffering capacity against 4-HNE toxicity in LV homogenates.

Remarkably, when comparing the relative changes of maximal Ca²⁺-dependent SERCA activities between the SERCA1a and SERCA2a isoforms, there were no significant differences in the degree of

impairments in response to 4-HNE treatment (Fig. 4C); observations which disagree with our hypothesis. However, these results could be interpreted differently in that SERCA2a displayed similar levels of inhibition even in the presence of higher reduced GSH and Hsp70 cellular content. In that context, SERCA2a may actually be more susceptible to 4-HNE mediated inactivation than SERCA1a which would be consistent with the general observation that SERCA2a is more susceptible to oxidation than SERCA1a (Viner et al., 1996). Initially, to directly test this hypothesis, plasmids containing either SERCA1a or SERCA2a cDNAs could be transfected into HEK-293 cells and cultured. The SERCA1a and SERCA2a containing HEK-293 cells could then be treated with 4-HNE and subsequently assayed for their respective maximal Ca^{2+} -dependent ATPase activities. If SERCA2a is more susceptible to 4-HNE inhibition, then it would be expected that there would be greater reductions in SERCA2a activity than SERCA1a activity when compared to their respective controls. Testing both isoforms in the same cellular niche overcomes limitations with respect to the cellular environment, as the cellular expression of GSH and Hsp70 would be consistent within both SERCA1a and SERCA2a expressing HEK-293 cells.

Summary and Conclusions

In summary, this study illustrates that 4-HNE inhibits SERCA1a and SERCA2a maximal Ca^{2+} -dependent ATPase activities independent of nitrosylation or glutathionylation of the SERCA pumps. 4-HNE treatment to WG and LV homogenates resulted in increased 4-HNE adduction to SERCA1a but not to SERCA2a, respectively. In addition, 4-HNE treatment resulted in significant increases in SERCA aggregation for both isoforms but reductions in FITC binding were only observed for SERCA1a. The predominant mechanism by which 4-HNE inhibits SERCA function is likely through destabilization and aggregation of the pumps; however, there are distinct isoform specific structural modifications caused by 4-HNE that likely contribute to functional impairments of the respective isoforms.

Limitations

There are a variety of limitations for this study. First, Western blotting techniques for protein carbonyl formation are non-specific, as standard DNPH and BH methods detect reactive carbonyl groups, 4-HNE, ACR, and MDA (Vasquez-Garzon et al., 2012). Therefore, due to this limitation, Western blotting was not recruited in measuring the

potential for enhanced ROS production and resulting SERCA inactivation with 4-HNE treatment. Secondly, Hsp70 expression and GSH content in WG and LV were not assessed in this study, so their role in explaining the differences between the SERCA isoforms are only speculative. Thirdly, we were unable to detect increased 4-HNE adduction to the SERCA2a isoform; therefore, our conclusions are limited as it cannot be definitively stated that 4-HNE directly adducted to and impaired SERCA2a function in our experiments. However, the inability to detect 4-HNE adduction to the SERCA2a isoform in our experiments may be a limitation with respect to the purification methods that were used. Lastly, there is currently no easy method to assessing the structural integrity of the high-affinity Ca^{2+} -binding region within the SERCA pump. 4-HNE is a small electrophilic and hydrophobic molecule; therefore, it is capable of diffusing through membranes which would allow it to potentially adduct onto the Ca^{2+} -binding region; thereby, causing structural destabilization and damage to the Ca^{2+} -binding sites and impairing SERCA function.

Future directions

With relevance to SERCA function, it would be of interest to confirm our hypothesis and identify if Hsp70 and GSH can protect against 4-HNE induced SERCA inactivation. As previously mentioned, this can be assessed by treating HEK-293 cells expressing either SERCA1a or SERCA2a with 4-HNE and subsequently assaying them for their respective maximal Ca²⁺-ATPase activities. Additionally, unpublished results from our lab indicate that sarcolipin (Sln), a micropeptide that binds within the transmembrane region of the SERCA pump, potentially protects SERCA from thermal inactivation. Therefore, it would be of interest to identify if Sln can also protect against 4-HNE induced SERCA impairment. This can be assessed by treating soleus muscles with 4-HNE from *WT* and *Sln*^{KO} mice and subsequently assaying their respective maximal Ca²⁺-ATPase activities.

Similar to free-radicals, 4-HNE rapidly oxidizes Cys residues (Petersen & Doorn, 2004; Schaur, 2003). However, this has yet to be demonstrated in SERCA pumps. It would be of interest to identify if 4-HNE significantly oxidizes Cys residues of the pump, which can be assessed by performing BIAM (N-(Biotinoyl)-N'-

(Iodoacetyl)Ethylenediamine) labeling studies. BIAM is a probe that labels free -SH groups, so reductions in BIAM labeling would be expected with 4-HNE treatment. Additionally, free-radical oxidation of Cys residues has been demonstrated to impair SERCA function; interestingly, studies have shown that free-radical SERCA impairment can be reversed through the addition of reducing agents. Therefore, it would be of interest to see if the addition of reducing agents such as dithiothreitol (DTT) can reverse the 4-HNE mediated SERCA impairments. Additionally, recruiting other methods of protein purification to establish 4-HNE adduction to SERCA2a would provide supporting evidence illustrating direct 4-HNE inhibition of the SERCA2a isoform. There is a growing trend towards the use of magnetic beads rather than sepharose beads in immunoprecipitation, due to their ease, reproducibility, and yield; therefore, using magnetic beads in subsequent experiments could potentially reveal increased 4-HNE adduction to the SERCA2a isoform. Also, it must be noted that the antibody used to detect 4-HNE adducts was directed against 4-HNE – His adducts; which are less reactive towards 4-HNE when compared to Cys residues. Therefore, using an antibody directed against 4-HNE – Cys residues

would be more beneficial when attempting subsequent experiments with the SERCA2a isoform.

SERCA impairment has been implicated in endothelial dysfunction and cardiovascular disease (Adachi et al., 2004; Cohen & Adachi, 2006; Hobai et al., 2013; Knyushko et al., 2005). Therefore, it would be of interest to identify if 4-HNE is involved in the pathophysiology of these diseases. This can be initially assessed by performing Western blots to see if there is increased 4-HNE adduction to SERCA2b and SERCA2a isoforms in endothelial and cardiovascular diseases, respectively. Furthermore, SERCA aggregation should also be recruited to confirm the mechanism of 4-HNE induced SERCA impairment in these disease models.

Appendices

Table A-1: Western blot antibody dilutions

Antibody Measure	Primary & Secondary Antibody Dilutions	
	Whole Homogenate	
	WG	LV
4-HNE-Histidine	1 ^o 1:2000	1 ^o 1:2000
	2 ^o 1:2000	2 ^o 1:2000
Nitrotyrosine	1 ^o 1:2000	1 ^o 1:2000
	2 ^o 1:2000	2 ^o 1:2000
Glutathionylation	1 ^o 1:2000	1 ^o 1:2000
	2 ^o 1:2000	2 ^o 1:2000
SERCA1a	1 ^o 1:10000	
	2 ^o 1:20000	
SERCA2a		1 ^o 1:2000
		2 ^o 1:2000
Antibody Measure	Immunoprecipitated	
	SERCA1a	SERCA2a
	WG	LV
4-HNE-Histidine	1 ^o 1:10000	1 ^o 1:10000
	2 ^o 1:20000	2 ^o 1:20000
Nitrotyrosine	1 ^o 1:10000	1 ^o 1:10000
	2 ^o 1:20000	2 ^o 1:20000
Glutathionylation	1 ^o 1:10000	1 ^o 1:10000
	2 ^o 1:20000	2 ^o 1:20000
FITC	1 ^o 1:5000	1 ^o 1:5000
	2 ^o 1:2000	2 ^o 1:2000

* All antibodies were diluted using 5% skim-milk with Tris-buffered saline plus 0.1% Tween20

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